Chapter 10
Diagnostic Tools for Plant Biosecurity

Jane E. Thomas, Thomas A. Wood, Maria Lodovica Gullino, and Giuseppe Ortu

Abstract There is now a wide range of diagnostic tools in the armoury to help prevent or control damaging disease outbreaks. When applied in the context of biosecurity, they have immense power to protect the plants on which food, feed, fuel and fibre supplies rely. Diagnoses which used to rely on culturing organisms, examining spores, or testing viruses on indicator plants, often taking many weeks to complete, can now be achieved in a matter of hours. Moreover, the advent of in-field diagnostic tests allows growers, agronomists or plant health and seeds inspectors to get a reliable test result without sending a sample to a laboratory. Remote sensing, using ground vehicles, unmanned aerial vehicles, or satellite technology, can bring a new dimension to surveillance, detection and diagnostic systems. Pathogen variation can be characterised rapidly by molecular marker techniques, potentially accelerating the process of identifying new pathotypes or fungicide resistant strains which threaten plant productivity. Metagenomic methods will undoubtedly play a part in non-targeted diagnostics, and identifying new threats to biosecurity. While diagnostic methods have advanced rapidly, their use in disease management in the field must be supported by robust sampling methods, treatment thresholds, and in depth understanding of disease risks.

Keywords Direct and indirect diagnostics • Molecular techniques • Pathotypes • Next generation sequencing • Remote sensing systems

J.E. Thomas (✉) • T.A. Wood
NIAB, Huntingdon Road, Cambridge CB 3 0LE, UK
e-mail: jane.thomas@niab.com

M.L. Gullino • G. Ortu
AGROINNOVA, Centre of Competence for the Innovation in the agro-environmental sector, University of Turin, Grugliasco (TO), Italy

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10.1 Introduction

Approximately 37 % of the earth's land surface is classified as agricultural land, that is land used for food production, including pastures and plantation crops. Natural or planted forests account for a further 30 % of land area. It is a major challenge to protect this area of vegetation from pests and pathogens in an era of global trade, commonplace international travel, and frequent severe weather events which can move disease propagules many hundreds of miles (Schmale and Ross 2015). The longer term effects of climate change will alter the distribution profile of many pathogens, creating new risks and reducing others (Pautasso et al. 2012) Traditional skills in plant pathology have declined in many developed countries, and surveillance resources have been severely stretched. New, or newly emerging, and established pathogens threaten productivity in many countries, affecting the food supply, livelihoods, and landscapes (Anderson et al. 2004). About 30 % of potential productivity is lost worldwide, pre-harvest, to the effects of pests and diseases, and a further 20 % may be lost post-harvest (Oerke et al. 1994). Pesticides, often the most effective method of control, are under pressure, both from regulatory requirements and rapid development of pathogen resistance. Plant breeding for resistance to pathogens can be very effective, but is relatively long term, and for many organisms there is rapid selection for the variants which render resistance factors ineffective. Prevention of new pathogen incursions, whether they are accidental, natural, or deliberate, is thus by far the most preferred approach, and rapid, accurate diagnostics are an essential component of preventative action. Plant biosecurity, defined by Waage and Mumford (2008) relates specifically to the protection of national boundaries against the introduction of alien pests and diseases. These organisms will normally be regulated and subject to statutory actions. However, other pest and diseases can be regarded as "emerging" threats, and have probably been present in a country or region for several years before first detection. For example, *Verticillium longisporum* was first confirmed on oilseed rape in the United Kingdom in 2007, (Gladders et al. 2011) and has now been found in over 20 % of the surveyed crop area (www.cropmonitor.co.uk). Early blight of potato, caused by *Alternaria solani*, once a rarity in Europe, has become prevalent in many European countries (Hausladen and Leiminger 2007), often necessitating specific spray programmes. The control of these, as well as long term established diseases, requires ever more sophisticated approaches to maintain profitable production, avoid unnecessary pesticide use, and still meet exacting quality standards, and the use of diagnostics in precision disease management will be increasingly required.

In the context of this chapter, the term biosecurity is used to refer to the prevention of incursions of alien pests or pathogens, by natural pathways or otherwise. However, since sophisticated diagnostic tools have advanced rapidly in the context of protecting crops and plants from plant pathogens in general, examples which relate to the management of indigenous diseases will also be discussed. The processes surrounding the application of diagnostics for biosecurity purposes, and some of the demands placed on a diagnostic method, *versus* those for disease

management may differ considerably. For instance, if a deliberate introduction is suspected, and forensic evidence for prosecution is needed, such as traceability to a source, appropriate accreditation standards will be needed. In some instances, precise strain or isolate discrimination will be needed, which may not always be required for indigenous disease management. For biosecurity purposes, the ability to detect extremely low levels of an organism is usually necessary, and quantification may not be needed. However, for disease management, accurate quantification is usually required, linked to treatment thresholds and economic relevance to a grower.

Diagnostic tools have to be deployed in a wide range of situations, and by a wide range of operators. These may include inspection services, growers, agronomists or staff in diagnostic service labs. In the context of regulated pathogens, training in the diagnosis of specific disease symptoms is usually available for inspection staff, and highly developed protocols, often using molecular techniques, are used for confirmation. Emerging or new, non- regulated threats in field crops are much more dependent on the observation skills of growers and agronomists, backed by "plant clinic" services, though a number of techniques such as remote or proximal sensing devices are increasing capability for wide area surveillance linked to a diagnostic imaging system of one type or another. Newer developments in molecular diagnostics such as loop mediated isothermal PCR (LAMP) and recombinase polymerase amplication (RPA) methods have brought specific and sensitive techniques for direct analysis of a sample within reach of field operations, giving rapid answers so that management actions can be put in place.

Diagnosis is the process of identifying a disease, and diagnostics describe the tools and tests used for this. Diagnostic tools may be considered in two broad categories. Firstly, those techniques where a sample can be viewed or analysed directly, and secondly, techniques where some remote or indirect analysis is involved. The first category is still the most common, and can cover everything from symptom recognition, immunological methods, and advanced molecular methods. The second category is less well established, but sophisticated technologies are increasingly offering a new dimension to diagnostics, incorporating wide area surveillance in the methodologies. This chapter will review some recent diagnostic approaches and show how they can contribute to plant and food biosecurity, and to the management of pathogens which continually threaten productivity.

10.2 Direct Sample Analysis

10.2.1 Symptom Recognition and Culturing

Diagnosis of a large number of diseases can often be achieved by visual inspection, either by eye on symptom type alone, or by observation of fruiting bodies with the aid of a hand lens or binocular microscope, or by culturing if appropriate and

examining mycelium and spore characteristics using compound microscopes. There are many high quality image libraries and descriptions available, such as the APS Press Compendium Series, and many on-line resources. Informative though these resources are, they usually show highly typical, clear and distinct symptoms. These can be very different to those a practitioner may see on a sample which has travelled in the mail, or which is at a different stage of development, or on a different part of the plant, or where other organisms or stress symptoms may be involved. Pattern analysis and machine learning of disease symptoms are techniques which are being developed (Camargo-Rodriguez et al. 2012, and Camargo-Rodriguez and Smith 2009) and provided symptom variability is incorporated, such systems could prove valuable in identifying diseases in areas where diagnostic support is not readily available and, importantly, perhaps to indicate when an expressed symptom does not match the range of known problems, and might be a new incursion. Web-enabled microscopy, allowing inter-laboratory examination of a specimen or culture, is available in several diagnostic networks (for example, the National Plant Diagnostic Network in the USA, www.npdn.org). Real-time consultation with experts while viewing a sample is a useful training mechanism as well as aiding diagnosis.

Simple techniques such as incubation in a damp chamber are useful for accelerating spore production, and can be used by growers, agronomists or laboratory staff. Early diagnosis of *Pyrenopeziza brassicae*, the causal agent of light leaf spot on oilseed rape, is essential to inform effective fungicide timings. The pathogen has a long latent period (Gilles et al. 2000) during which symptoms are absent or very unclear, and can easily be confused with frost damage, fertiliser scorch or possibly other diseases. However, where infections are present, incubation of a leaf sample in a polythene bag at about 18 °C for 3–4 days, will reveal the presence of the spore producing acervuli, which are easily visible to the naked eye.

Culturing from an infected sample to obtain putative causal organisms for re-inoculation and the proof of Koch's Postulates is of course a standard procedure, and there is a multitude of modified substrates, selective agars, surface sterilisation and incubation conditions which can be used to try and identify primary causal agents. In the Plant and Food Biosecurity programme, a long term study of the onion- *Fusarium proliferatum* pathosystem was initiated after the pathogen was identified in cultures from diseased onion bulbs in Israel in 2008. A selective medium was developed which encouraged growth of *F. proliferatum* over non-target fungi present in the sampling environment, and which enhanced the development of distinctive spore types which differentiate *F. proliferatum* from other Fusaria (Isack et al. 2014). Highly selective diagnostic media such as this are particularly valuable in labs where molecular diagnostic facilities may be limited.

The Plant and Food Biosecurity project has developed a virtual diagnostic network, described in detail elsewhere in this book, which can act as a repository for the types of information described above, including methodologies and outbreak mapping, expert finding, the potential for web-enabled microscopy as an add-on facility, community pages for protocols, and diagnostic training.

10.2.2 Molecular Diagnostic Methods

Despite the effectiveness of traditional diagnostic skills in many instances, they are usually lengthy and while they are ongoing, a potential disease threat may be increasing in the environment. Some pathogens are obligate, and some threats will occur with sub-species, pathotypes, or other variants which cannot be distinguished from an established problem visually or in culture. Viruses are particularly difficult to diagnose visually and symptoms may vary according to cultivars of the host species as well as virus strain. Enzyme Linked Immunosorbant Assays (ELISA) have long been the basis for plant virus diagnosis. Boonham et al. (2014) suggested that high levels of reproducibility, repeatability. the availability of an industry standard format and easy access for most laboratories were all reasons why the technique remained successful and widely used.

Immunological assays have been developed into lateral flow devices for on-site pathogen detection, for viruses, bacteria and some fungi. The kits are of use to both regulatory officials and growers or agronomists, and give rapid answers, though for testing multiple samples, their cost is relatively high. As with other targeted test methods, LFDs can only answer the specific question of whether a known organism is present, and low sensitivity can limit their value. However, they are increasingly used as on-site diagnostics by growers and agronomists, and have significant value for early detection of disease threats, and are available for virus, bacteria, oomycete and fungal targets. LFDs have been developed to diagnose the presence of *Rhizoctonia solani* in soil (Thornton et al. 2004). Wakeham (2015) developed an LFD for the detection of the club-root pathogen in commercial brassica growing soils. Though the accuracy of the LFD was lower than a qPCR test for disease prediction, it could be used for rapid indexing of soils, in 10 min, by growers to indicate whether the risk of club-root was zero or low, or medium to high. Kennedy and Wakeham (2008) developed monoclonal antibodies against the onion downy mildew pathogen, *Peronospora destructor*, and devised a lateral flow format for the detection of sporangia. Lane et al. (2007) evaluated LFD assays for two Phytophthora species associated with tree diseases and concluded that they compared favourably to a PCR diagnostic and far exceeded visual accuracy. They were considered suitable for phytosanitary purposes, and identifying samples for more stringent analyses. The Phytophthora kits may also be used for detection of late blight on potatoes and tomatoes. LFD reader systems have also been developed, allowing for semi-quantitative interpretation (Faulstich et al. 2009), thus adding value to the assays in terms of the degree of risk which may be predicted. LFDs have the advantages of being easy to use, rapid, and can be applied to many types of plant tissue which can be extracted on site, including woody plants. However, cross reaction with non-target species, delayed or weak reactions can result in misleading information, and potentially wrong management decisions being made. Unless specificity is very high, such as with LFDs developed for detecting potato viruses, the use of the devices as on-site diagnostic may be limited. The majority of commercially available

LFDs are for viruses, with some bacterial and relatively few fungal or oomycete targets. Multiplexing is rare, and available assays target one organism only.

10.2.3 Air Spora Diagnostics

The use of spore traps for detecting the presence of a pathogen can play a major role in monitoring the environment for new incursions and also management of established pathogens. Types of trap samplers have been extensively reviewed by West and Kimber (2015). In the past, spore traps have required a lengthy period between time of sampling and the point at which a quantitative figure for the presence of a target pathogen in the air could be given. Microscopic examination of tapes and strips required a very high level of mycological expertise. However, grower-operated Burkard spore traps can now be used in conjunction with LFDs for analysis of the sample. The Brassica Alert system (www.syngenta-crop.co.uk/brassica-alert) offers the option of an LFD assay for the brassica ringspot fungus (*Mycosphaerella brassicicola*). Early detection of the spores offers the opportunity for early intervention with fungicide and prevention of lesion development, since the retail standards for blemish in vegetable brassicas can be particularly stringent in many European countries. In the context of biosecurity, the analysis of spore trap catches could be very valuable for tracking incursions. However, Jackson and Bayliss (2011) reviewed the use of spore traps for biosecurity objectives, and concluded that their application was constrained by the relatively small volume of air that could be sampled, and the difficulty of achieving adequate representation of the air spora of a region. Analysis time was considered to be less of an issue given new diagnostic approaches.

Direct analysis of a spore trap sample by qPCR is possible, though this still requires a laboratory test. It has the advantage that tests can be multiplexed, and a range of targets from a single spore trap sample can be quantified. Isothermal assays could be conducted in the field, with answers available in a few minutes. Thiessen et al. (2016) used an isothermal assay to detect spores of grape powdery mildew from impaction spore traps placed in vineyards. The data was used to alert growers on spray schedules, and by using the system they were able to reduce spray number compared to calendar driven spray schedules, without affecting disease severity. West et al. (2013) developed an assay to quantify Sclerotinia risk for oilseed rape crops. Spores from an impactor trap were captured in vials containing a semi-selective medium. This was then testing for the presence of oxalic acid, secreted by ascospores of *S. sclerotiorum*, using a biosensor, and the results wirelessly transmitted to a server. Together with on site weather recording, the risk of Sclerotinia was evaluated and sent to the grower *via* text message. Fully integrated trap and diagnostic systems, using LFDs, isothermal PCR methods or other analyses are being developed further, and potentially can give local, regional or national risk indications depending on siting, and type of trap used. The concept of mounting spore traps on lower atmosphere UAVs, or even higher altitude systems, in combination with a diagnostic system, could increase sampling capacity and give new insights

into mass spore movements, and the possibility of predicting pathogen outbreaks or incursions well in advance of disease symptoms being discovered.

10.2.4 DNA and RNA Diagnostics on Plant Material and Soil

The rapid development of nucleic acid detection systems in plant pathogen diagnostics has given rise to many standardised and validated protocols. For regulated pathogens, the European and Mediteranean Plant Protection Organisation (EPPO) publishes new diagnostic procedures, and several of these now contain PCR based methods. The International Seed Testing Association (ISTA), and the International Seed Health Initiative (ISHI) have also validated a number of molecular diagnostic protocols to establish seed health status. Apart from regulated materials and quarantine pathogens, many other studies have resulted in the production of nucleic acid based methods which can be used to diagnose pathogens rapidly and accurately, and contribute to more effective disease management. PCR, qPCR and RT-PCR are now standard laboratory diagnostic techniques, and have been reviewed extensively over the last 10 years (see Atkins and Clark 2004; Bradshaw et al. 2006; Martinelli et al. 2014). Signifcant advances in the use of PCR diagnostics for soil-borne pathogens, and other problematic substrates, have been made. Woodall et al. (2012) developed a quantitative PCR test to detect sclerotia of the onion white rot pathogen (*Sclerotium cepivorum*) in soil. Bilodeau et al. (2012) developed an assay to detect microsclerotia of *Verticillium dahliae* from soil samples in strawberry growing fields. Deora et al. (2015) used a Taqman PCR assay to detect and quantify club-root (*Plamodiophora brassicae*) from soil samples. These, and other examples, typify situations where conventional mycological tests take extended periods to produce results, or require complex extraction and processing, with the need for highly experienced staff to count propagules. They also represent serious pathogens of high value crops, where avoidance of infected land is the principle means of control. Several commercial soil extraction kits are now available and the cost of diagnostic services using them should be acceptable to growers.

10.2.5 Early Detection of Emerging Diseases in High Value Crops

Vegetable and salad crops are usually produced in relatively small, specific areas of a country, in close rotation, and often with two or more crops produced in a single season. These intensive growing conditions are very favourable for the rapid increase of disease. If pathogens are carried on or in seeds, even very low levels of contamination can lead to the rapid emergence of new disease problems which threaten crop quality and saleability (Gullino et al. 2014). The development of

diagnostic tools for early detection and investigation of causal agents is essential to support prevention and control measures.

Case Study 1 – Wild Rocket and Lettuce Diseases
Two recently occurring diseases on wild rocket and lettuce in Italy were investigated as part of the Plant and Food Biosecurity project. *Plectosphaerella cucumerina* was described as the causal agent of a leaf spot on wild rocket (*Diplotaxis tenuifolia*) in Italy in 2012 (Garibaldi et al. 2012). The disease was severe under glasshouse conditions and caused significant production losses, with rotting of material occurring after processing and packing. A real-time PCR assay for the detection of *Pa. cucumerina* was first developed by Atkins et al. (2003). However, there are four *Plectosphaerella* species, and analysis of the sequence data suggested that the assay was not specific enough to distinguish between these. Specific real time Taqman probes were therefore designed targeting areas of the ITS sequence with most divergence between species to detect and quantify *Pa. cucumerina*. This assay was able to detect *Pa. cucumerina* DNA in symptomatic rocket leaf tissues and was shown to be highly specific, with no amplification of non-target species of *Plectosphaerella*.

Fusarium. oxysporyum f. sp. *lactucae* is the causal agent of wilt on lettuce (*Lactuca sativa*). It has spread through many countries and was first detected in Europe in Italy in 2002 (Garibaldi et al. 2002) causing up to 70 % losses in summer production. Three different races (1, 2, and 3) have been identified within *F. oxysporum* f.sp. *lactucae.* Currently only race 1 has been reported in European countries (Garibaldi et al. 2002; Gullino et al. 2004) while races 2 and 3 have been only reported in Japan (Fujinaga et al. 2001, 2003) and Taiwan (Lin et al. 2014). Pasquali et al. (2007) developed an assay based on inter-retroelement amplified polymorphism (IRAP) PCR which differentiated *F. oxysporum* f.sp. *lactucae* Race 1 from other isolates of the pathogen, and other *F. oxysporum formae speciales* This assay could be applied to both plants and seeds for rapid detection of Race 1 of the pathogen, thus supporting disease management and use, or not, of seed stocks.

10.2.6 Multiplex Diagnostics

Multiplex detection of several pathogens in a single assay is still relatively limited, though many target organisms are either part of a disease complex, or cause distinct diseases while existing in the same sample substrate. Cullen et al. (2000), developed a qualitative multiplex PCR test for three potato pathogens in soil and on tubers. Qu et al. (2010) used a TaqMan PCR for simultaneous detection of powdery scab and common scab on potato tubers. Visual discrimination of symptoms of these two diseases can be difficult, but is necessary for effective management of seed tubers. Recently, the Luminex® platform has provided a new mechanism for multiplexing nucleic acid or protein based assays. In the case of nucleic acids, there is specific hybridisation between DNA fragments on colour coded paramagnetic beads.

Kostov et al. (2015) used Luminex® technology to detect and identify 26 species of Phytophthora simultaneously. A further 22 samples were identified to clade or subclade levels. Discrimination at this level provides a significant advance for detecting closely related organisms which have major phytosanitary implications throughout Europe and elsewhere. Charlermroj et al. (2013) used Luminex® technology for the simultaneous detection of three plant viruses and a seed- borne bacteria. The assay detected all pathogens from infected plant material, and could be adapted to detect up to 50 targets at the same time. Though laboratory based, these multiplex systems offer considerable efficiencies which will assist the safe movement of plants and seeds across national borders.

10.2.7 On Site, Field Deployable Diagnostics

Despite the speed and sophistication of new laboratory based molecular diagnostics, on-site diagnosis is still a practical requirement for both regulated and non-regulated pathogens (De Boer and Lopez 2012). For regulatory organisms, rapid diagnosis is usually needed at the point of inspection, so that consignments can be moved onwards or held back (Brasier 2008). For non-regulated pathogens, where effective disease management is at stake, growers and agronomists frequently need a rapid diagnosis to select an optimal spray programme, including appropriate active ingredients and rates. The use of LFDs as previously discussed has already made an impact, and on-site DNA diagnostics is now a rapidly developing area. Loop mediated isothermal PCR (LAMP) operates at between 60 – 65 ° C and does not require a thermal cycler. Isothermal assays can be carried out on crude extracts from target tissues, with no DNA purification steps, and can operate effectively in the presence of many inhibitors which are usually present in plant, seed or soil samples. Developed assays and closed tube systems are available (see www. optigene.co.uk) marketed with Genie II or Genie III instrumentation. These units are portable and robust, running for a day on battery power, and delivering easy to interpret visual assay results within 15–30 min. Other isothermal diagnostic systems have been developed, and are now being investigated in the plant health arena. Miles et al. 2015 used recombinase polymerase amplification (RPA) to detect Phytophthora species, and suggested its use as field deployed diagnostics for Phytophthora infections of forest trees. The RPA assays were very rapid, and as with LAMP did not require DNA purification. Doan et al. (2014) used RPA to diagnose *Fusarium oxysporum* f.sp. *vasinfectum* race 4 in cotton plant tissues. The assay was race specific, and was carried out at a constant 39 °C, giving a result in 30 min. Identifying Race 4 infected fields is important for the prevention of spread of the race *via* seed and soil. In the growing region of California, where the work was carried out, seed and ware production of cotton takes place in the same area, so restricting infection to as few fields as possible is necessary. Ultimately, highly sensitive techniques may be able to detect target regions of DNA without any amplification step. These approaches

are already being developed for medical applications (Cai et al. 2015) as point of care diagnoses.

The on-site diagnostic systems currently available in the plant health sector are single target assays, so several tests may need to be performed on a single sample. Nevertheless, they are providing much needed efficiencies, and new information to feed into disease prevention and control strategies.

10.2.8 Biosensors

Biosensors offer a novel and potentially very efficient method of diagnosing plant pathogens. They consist of a biological molecule (protein, nucleic acid, enzyme etc) which can specifically recognise a target analyte. The reaction is then converted to a signal by a physiochemical mechanism (the transducer). Biosensors could be deployed in large numbers in agricultural environments, or in produce storage systems, to detect the presence of pathogens or rots. Signals could be captured wirelessly and transmitted to a server in the farm office or to a store manager, or integrated with a disease forecasting system, to create alerts and prompt action. Fang and Ramasamy (2015) reviewed the potential for biosensors in plant disease detection. Though biosensors for fungi, bacteria, oomycetes and viruses had been developed in the laboratory, their practical use as an in- field detection systems has not yet been realised. They concluded that enzyme based biosensors probably had the greatest potential as they were stable, relatively low cost, easy to use and specificity could be very high.

10.2.9 Non-targeted Diagnostics

A major challenge facing diagnostics is the presence of an "unknown" threat. Next generation sequencing technologies, and the bioinformatic systems which accompany them, are being deployed to detect new causal agents without any prior knowledge of their identity. Simply, sequencing extracts from a plant expressing symptoms and comparing it to sequence data from a plant with a healthy appearance, can reveal differences which point to potential causal agents. Adams et al. (2009) identified a previously unknown cucumovirus using this approach, and Barba et al. (2014) in a review of the techniques involved, itemised the discovery of viruses or viroids in tissues of many plant species that exhibited unknown disease etiologies. They suggested that NGS technologies were a future tool in quarantine and certification of high value fruit species and in woody plants where virus titres may be very low.

Nanopore sequencing is a third generation sequencing technology generating long read lengths. While relatively new to plant pathology, it is already being used in metagenomic studies to identify human bacterial and viral infections

(Greninger et al. 2015). The technology identifies nucleotides in a DNA molecule by measuring conductivity as DNA molecules pass through pores in a membrane. The MinION™ device is a portable pocket sized unit containing disposable flow cells and which can be plugged into a PC *via* a USB port and results read in real time on the screen. Multiple re-sequencing of pathogen isolates and comparison with reference genomes could aid understanding of genetic variation and virulence changes, and metagenomic sequencing of plant, soil or air samples could identify emergent problems before they become established. While the technology is still in very early stages of research for plant pathogens, it offers great potential for future precise understanding of microbial diversity, and the new threats that may exist.

10.2.10 Diagnostic Techniques for Detection of Novel Pathotypes

The discrimination of pathogen strains or pathotypes is a major factor in plant biosecurity. Though pathogens may be well established threats in particular areas, and the diseases caused easy to recognise by symptoms alone, the evolution and selection of new virulences which defeat previously effective host resistance factors, can frequently cause sudden and serious disease outbreaks. One of the most common examples are the rust pathogens of cereals and other crops. Long distance transport of spores, either by extreme weather events or inadvertently by humans during international travel, is probably responsible for the movement of pathotypes common in one region to a different area. However, rust pathogens have also been weaponised in the past (Rogers et al. 1999) so the potential for deliberate introduction exists.

The diagnosis of pathotypes of common, established diseases is still achieved largely by field sampling and testing on host differential lines which reveal variation in virulence matching host resistance genes or factors. For wheat rust pathogens, many countries worldwide carry out regular surveys of field outbreaks from commercial crops, or selected sentinel plots which remain unsprayed and expose different resistances to try and detect early occurrences of new virulences. Despite these efforts, there is often a considerable time lag before confirmation of new pathotypes, particularly when field testing on adult plants is required as well as seedling tests. Extensive facilities and staff resources are also needed, and the sampling intensity of outbreaks is inevitably restricted. Frequently, new pathotypes are not detected at very early stages of establishment, with the result that previously resistant cultivars can suddenly "break down" in the field. Marker techniques such as simple sequence repeats (SSRs) have proved effective in tracking programmes for rust populations and associated pathotypes, and understanding global pathogen evolution (Hovmoller et al. 2015). Nevertheless, such systems still require careful increase of these obligate pathogens on host plants, which can take many weeks.

Case Study 2 – *Puccinia striiformis* **f.sp**. *tritici*
Recently, the concept of field pathogenomics has been developed to obtain more rapid information on the occurrence of new virulence phenotypes (Hubbard et al. 2015). Using the wheat yellow rust pathogen (*Puccinia striiformis* f.sp. *tritici*) in the U.K. as a model, the technique employed transcriptome analysis of field samples collected in tubes of RNAlater. A section of the same infected leaf was reserved to increase the rust spores and carry out conventional pathotyping on host differentials. Analysis of the genetic structure of the samples showed that four distinct lineages were present, which corresponded to distinctive virulence phenotypes. The method was able to show that the yellow rust population had recently undergone a major change genotypically, with a highly diverse structure compared to historical isolates. Together with field observations of a higher than usual incidence of teleutosori, the evidence pointed to an exotic incursion, probably from a region where the pathogen underwent a full heteroecious cycle on its alternate host. The technique also created the potential for greatly increased sampling intensities at a relatively low cost, and prediction of which samples appear unusual genotypically, and should be taken forward for standard phenotypic analysis. Rapid genotyping techniques for diagnosing new races of stem rust (*Puccinia graminis* f.sp. *tritici*) have also been developed using a SNP chip (Singh et al. 2015). The field pathogenomics concept, though laboratory based, offers very considerable scope for the rapid characterisation of emergent pathotypes, particularly for obligate organisms, and so contributes to enhanced biosecurity through speed of response and re-direction of breeding programmes.

10.3 Indirect Diagnostic Methods

10.3.1 Disease Diagnostics by Remote or Proximal Sensing

The vast areas of cultivated plants that may be at risk of pathogen attack cannot be adequately monitored by the standard method of crop walking and visual inspection, though these tried and tested methods will always be needed, both for identifying regulated pests at ports of entry, and for the management of indigenous diseases. Halting epidemics or spread of a pathogen threat is most effective at very early stages of development, and for this different approaches are needed. Aerial photography has long been available for locating foci of a range of diseases, though confirming identity of the specific problem has been achieved by site visits. Now, ground vehicles, UAVs, and satellite platforms, can all be equipped with various types of sensing equipment which can identify unhealthy plants, creating the potential for wide area monitoring. Mahlein (2016) reviewed the use of disease detection by imaging sensors and concluded that there was as yet no commercially available system which could be used for the specific detection of plant disease. The recognition of specific diseases, and discriminating between them, and other causes of plant

stress, with sensing technologies, remains a major research challenge, though some experimental progress has been made.

Measuring reflectance from plants in the field in the elcctromagnetic spectrum from multispectral or hyperspectral cameras holds significant promise for disease detection. The most informative spectral bands range from visible though to infrared and thermal wavelengths. Passive remote sensing devices measure reflectance of incident radiation from the plant canopy, while active sensors emit radiation to the target and measure returned reflectance. The collection of multiple spectral information, at as high a resolution as possible, may start to build up "signature" information for specific disease or pest infections, but extensive ground truthing is a critical aspect for research. There is also a significant image processing and computational requirement before positional data on "affected" versus "healthy" plants can be mapped onto field layouts.

Information from different areas of the electromagnetic spectrum will have different functionalities. Visual wavelengths are unlikely to detect pre-symptomatic infection, but will be important in tracking disease epidemics and providing new insights into disease dynamics. Early detection or pre-symptomatic disease is more likely to be achieved by multispectral or hyperspectral images. Bauriegel et al. (2011) identified Fusarium head blight infection in wheat using hyperspectral imaging under laboratory conditions. However, they further identified two specific and narrow wavelength ranges which could be used under field conditions and defined the optimal growth stage for predicting infection levels. Huang et al. (2007)) used air-borne hyperspectral imaging to detect wheat yellow rust, and obtained a high correlation with ground assessment of a disease severity index. Specific spectral features were associated with yellow rust infection, but not with water or nutrient stress.

Wahabzada et al. (2015) used hyperspectral reflectance to identify specific signatures of foliar diseases of barley at different stages of development, and coined the term "metromaps" to describe them. Though the material was inoculated and assessed under laboratory conditions, such specific hyperspectral signatures could possibly be used to identify field outbreaks of diseases before complete symptom development, enabling precision application of eradicant fungicides.

UAVs, either fixed wing or rotary platforms, have the ability to survey large areas, though suffer from several drawbacks. Licences to operate may be restricted in some areas, and payload will be limited for rotary type systems. Though more can be carried by fixed wing UAVs, these are more expensive and operators require more training, with higher standards and more restrictions. The frequency of flights can thus be limited, and insufficient to enable the build up of images which might indicate a spreading disease. Satellite technology has the advantage of more frequent image collection, continuing improved resolution, and relatively inexpensive access. Cloud cover and other environmental conditions can restrict operational periods. However, to date, applications are mostly in the surveillance of crop type and planted areas rather than within field diagnosis of potentially diseased plants and there are few examples of remote sensing of specific diseases from satellite platforms. Mirik et al. (2013) mapped outbreaks of wheat streak mosaic virus in

Texas using reflectance data gathered by the Landsat 5 satellite. Yuan et al. (2014) detected wheat infected with powdery mildew using multispectral imaging from the SPOT 6 satellite with a maximum of 89 % accuracy. For the medium term however, it is probable that satellite sensing for disease diagnostics is more likely to be used to identify areas of stressed plants, where ground confirmation of specific causal agents is needed.

The detection of volatile organic compounds (VOCs) is also an indirect diagnostic method, aiming to detect specific signatures of volatiles that a host plant emits when challenged with a pathogen. Many factors may affect the volatile signature of a plant-pathogen combination, including the age of the plant, non-disease related stress, nutrient status etc. A wide range of control plants must therefore be investigated in order to separate disease associated volatiles reliably. A number of electronic nose (EN) systems have been developed. Ghaffari et al. 2012 used 13 gas array sensors to discriminate successfully between healthy and pathogen or pest challenged leaves of tomato, cucumber and pepper, and concluded that the EN system would be effective for diagnosing disease in commercial glasshouses. FAIMS (field asymmetric ion mobility spectroscopy) is an alternative to EN technology. Both methods use fingerprinting of volatile biomarkers, but FAIMS uses mobility of molecules rather than a chemical detection of compounds. It is thought to be more sensitive, and Rutolo et al. (2014) reported successful application of FAIMS profiling to detect bacterial soft rots in potatoes under laboratory conditions. Aksenov et al. (2014) recently identified a specific VOC profile of citrus plants infected with the citrus greening disease (*Candidatus Liberibacter*). The assay was very accurate, even at early pre-symptomatic stages, and could be deployed in the field for growers as a portable device with further refinement. VOC profiling in this case has great potential for critical early identification of a devastating disease, so that infected trees can be removed.

Obtaining pathogen specific profiles for VOCs remains a research challenge in the majority of cases (Sankaran et al. 2010), though detecting healthy versus generically challenged plants may suffice in many situations, such as removing rots from storage environments.

10.4 Conclusions

The range of pathogen diagnostic tools, and the level of their sophistication, has never been greater, and with the advent of new sequencing techniques and novel, field deployable diagnostic systems, their potential can only increase. Maintaining plant biosecurity in the area of regulated pathogens will be a clear beneficiary of these developments. Recent occurrences of new forest and amenity tree pathogens in many countries has brought an unprecedented level of awareness of plant pathology to the general public. In crop plants, cereal rust fungi, citrus greening disease, downy mildew of basil, and the *Xyllela fastidiosa* infections of olive trees have made recent national headlines in different countries. This new awareness brings

advantages in that well informed crowd-sourced observations can help to monitor and track disease outbreaks, and potentially discover unusual occurrences, at a level that research organisations and inspection services alone could not achieve. It has also brought investment, both public or private, in developing diagnostic systems. To some extent, it has revitalised interest in plant pathology, and the existence of a skills gap has been acknowledged in many countries, though there is still significant effort needed to ensure that skills can be taught and acquired by new generations of pathologists.

In the regulated pathogen sector, the simple aim of diagnostics must be to identify the causal agents of disease accurately even when levels are very low, and thus contribute to their exclusion, or containment. Detecting and diagnosing "unknown" threats with metagenomic analyses brings a new level of protection against threats to biosecurity. Strain, or pathotype discrimination is important to detect continually evolving variants of pathogen species. Diagnostic tools themselves require validation, and service providers need ongoing training, participation in reference testing and maintenance of international accreditation. Where indigenous organisms are concerned, diagnostic systems face different challenges. While accurate identification is still an obvious pre-requisite, the whole disease management system has to be taken into account. Sampling techniques, weather conditions, treatment thresholds, product availability, rotational decisions, cultivation options, seed bed conditions, cultivar choice, end use requirements, likely price for a crop, are all part of the decisions that need to be made when particular diseases are diagnosed, and a diagnostic tool needs to give results which are readily interpreted in the overall context of a crop situation. Practicalities need to be considered as well. For instance, growers frequently need to apply agrochemical for disease control regardless of risk, or at times which are not optimum, because of farm workloads, contract sprayer schedules, and weather. In some cases, prophylactic spraying is preferred rather than risk determined sprays because quality requirements are so high, and failure to meet them can result in significant loss of income. Countering this however are increasing restrictions on agrochemical use, loss of active ingredients, and low to zero residue level requirements. Diagnostics has a role to play in the effective targeting of control, by integrating with smart spray systems linked to GPS which only apply product where needed. Precision farming, and the drive for sustainable intensification, will place more demands on diagnostic systems, both field deployable units and rapid, sophisticated laboratory methods, all linked with a thorough understanding of the epidemiology of disease causing organisms.

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